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PREPARATION AND PROPERTIES OF ORGANOPHILIC TRYPSIN MACRO-INHIBITORS: DIAMIDINO-α,ω-DIPHENYLCARBAMYL-POLY(ETHYLENE GLYCOL)

Georges TAKERKART*, Emile SEGARD** and Michel MONSIGNY*†

* *Centre de Biophysique Moléculaire, C.N.R.S. et Laboratoire de Chimie Biologique, UER Sciences 45045 – Orleans-Cedex, France and ** Centre d'Etudes Cryogéniques de l'Air Liquide 38360 – Sassenage, France*

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1. Introduction

Aqueous two-phase systems are largely used to purify biological macromolecules [1]. One of the most frequently used systems is obtained by mixing aqueous solutions of poly (ethylene glycol)* (PEG) and dextran. Better results were obtained in this system upon introduction of poly (ethylene glycol) carrying covalently bound charged groups [2]. Conditions for obtaining optimal separation of glycolytic enzymes of yeast lysate by this procedure have been investigated by Johansson et al. [3]. However, good separation requires the use of counter-current distribution.

It would be of interest to investigate how specific ligands bound to PEG, affect the partition of proteins. For this purpose, p-aminobenzamidine PAB, a strong inhibitor of trypsin, was attached to PEG of different molecular weights. The preparation and some of the properties of these new trypsin inhibitors are described here.

2. Materials and methods

PEG 200 was obtained from Koch-Light. PEG 2000 and PEG 9000 were obtained from Fluka. Trypsin, PAB and DL-benzoylarginine *p*-nitroanilide (BAPNA) were supplied by Sigma.

PEG 200, 2000 and 9000-α,ω-dichloroformiates were prepared at room temperature by reaction of phosgene with PEG in toluene [4].

Absorption spectra were recorded with a Unicam SP 800 spectrophotometer. Extinction coefficients were determined using a Zeiss PMQ II spectrophotometer. Infrared spectra were recorded with a Perkin–Elmer model 257 spectrometer. Melting points were determined on a Leitz apparatus and were not corrected. Refractive indexes of chromatography column effluent were determined with a Zeiss refractometer. Purity of compounds was tested by gel filtration using columns of Sephadex G 25 or G 75.

Trypsin activity was determined according to Erlanger et al. [5] and Mares-Guia and Shaw [6] using DL-BAPNA as substrate. After addition of the enzyme to the sample cuvette, the absorbance changes were recorded at 410 nm for 3–4 min; the slopes of the straight lines obtained gave the initial rates of reaction.

K_i values were determined according to Dixon [7].

2.1. *Preparation of diamidino-α,ω-diphenylcarbamyl-poly (ethylene glycol) 200, 2000 and 9000 (PEGPAB)*

p-Aminobenzamidine hydrochloride (350 mg; 2 mM) was dissolved in 20 ml of 1 M $NaHCO_3$ solution. PEG 200-α,ω-dichloroformiate (320 mg; 1 mM) was added in 5 fractions in 30 min.

After 20 hr of continuous stirring at room temperature, the mixture was adjusted to pH 3 by addition of 5 N HCl and then evaporated to dryness in vacuo. The residue was extracted with anhydrous

† To whom inquiries should be addressed.

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methanol and the extract was concentrated to a small volume. Upon addition of ether, the product was precipitated (yield : 300 mg; 45%). The compound turned brownish without melting when heated above 280°C.

IR(KBr)cm^{-1} : 2900 (alkane), 1735 (amide I), 1680 and 1610 (amidine), 1525 (amide II), 1150 – 1050 (ether). UV : $\lambda_{max}^{H_2O}$: 270 nm: $E_{1\,cm}^{1\%} = 380$.

PEG 2000 PAB and PEG 9000 PAB were obtained in a similar way but the solution was stirred for 48 hr and the purification was carried out in the following way. The methanolic extract was concentrated to dryness and there sidue taken up in anhydrous tetrahydrofuran. Ether was added to the filtrate and the solution allowed to stand at −10°C for 2 hr. The white material obtained was collected by filtration, washed with anhydrous ether and dried in vacuo.

PEG 2000 PAB: yield : 68%; m.p. 51°C

UV: $\lambda_{max}^{H_2O} = 270$ nm; $E_{1\,cm}^{1\%} = 82$.

PEG 9000 PAB: yield : 80%; m.p. 61°C

UV: $\lambda_{max}^{H_2O} = 270$ nm; $E_{1\,cm}^{1\%} = 16.5$.

3. Results and discussion

3.1. *Inhibitor characterization*

The structure of the prepared compounds was proved by infrared spectroscopy, UV-absorption, gel chromatography and determination of inhibition constants.

First, it appears from IR and UV spectra that PAB has been covalently bound to PEG by its primary amino group. IR spectra show an absorption at 1735 and 1525 cm^{-1} assignable to > CO of a carbamate group. They also exhibit a strong absorption at 1680 and 1610 cm^{-1} assignable to the amidine function. No absorption can be detected in the 1330–1340 cm^{-1} range, showing the absence of primary aromatic amino group. UV spectra of the compounds in water show an absorption maximum at 270 nm whereas the UV spectrum of PAB exhibits a strong absorption at 290 nm (fig. 1). This blue shift is related to the substitution of the amino group of PAB.

Secondly, from the gel filtration and the melting points of the compounds, it may be concluded that the molecular weights of PEGPAB and of original

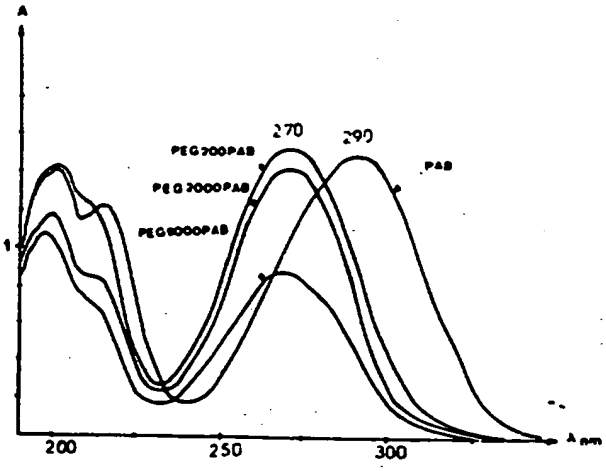

Fig. 1. Absorption spectra of PAB (17 μg/ml), PEG200PAB (62 μg/ml), PEG2000PAB (192 μg/ml) and PEG9000PAB (660 μg/ml) in aqueous solution.

PEG are almost identical. An elution diagram of PEG 9000 and PEG 9000 PAB on Sephadex G-75 column is shown in fig. 2.

Finally, the rate of hydrolysis of BAPNA by trypsin decreases sharply when PEGPAB is present in the medium indicating strong inhibition.

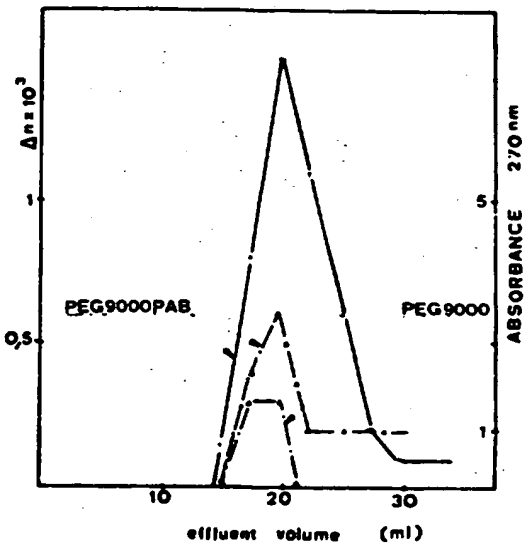

Fig. 2. Elution diagram for the PEG9000 and the PEG9000PAB on a 1 × 50 cm column of Sephadex G-75, 0.05 M phosphate buffer pH 7.0. +—+—+—+ absorbance at 270 nm; ▲—▲—▲—▲ refractive index.

3.2. *Kinetic properties of inhibitors*

For the evaluation of the K_i values of PEGPAB with DL-BAPNA as a substrate, a kinetic treatment was developed which takes into account the presence of two inhibitory sites.

The system can be described by:

$$E + S \rightleftarrows ES \rightarrow E + P$$
$$E + I \rightleftarrows EI$$
$$EI + E \rightleftarrows E_2I.$$

where the dissociation constants are:

$$K_s = \frac{(E)(S)}{(ES)} \quad (1)$$

$$K_i = \frac{(E)(I)}{(EI)} \quad (2)$$

$$K'_i = \frac{(EI)(E)}{(E_2I)} \quad (3)$$

The concentration of ES is given by the following equation:

$$p^2 \frac{K^2_s}{s^2} \frac{i}{K_i \cdot K'_i} + p\left(1 + \frac{K_s}{s} + \frac{K_s}{s}\frac{i}{K_i}\right) - e = 0, \quad (4)$$

where p is the concentration of ES, i that of I, s that of S and e the total concentration of E. Assuming that

$$4\frac{K_s^2}{s^2} \cdot e << \frac{K_i K'_i}{i}\left[1 + \frac{K_s}{s}\left(1 + \frac{i}{K_i}\right)\right]^2 \quad (5)$$

the equation (4) can be solved easily, we get

$$p = \frac{e}{1 + \frac{K_s}{s}\left(1 + \frac{i}{K_i}\right)} \quad (6)$$

and

$$v = \frac{V}{1 + \frac{K_s}{s}\left(1 + \frac{i}{K_i}\right)} \quad (7)$$

Eqs. (6) and (7) are those obtained in the case of an inhibition by a mono-inhibitor. Therefore, Dixon's graphical method can be used to obtain the K_i values.

The experimental data are shown in fig. 3. The K_i values of the three PEGPAB are not very far from that of PAB itself. This may be explained by the fact

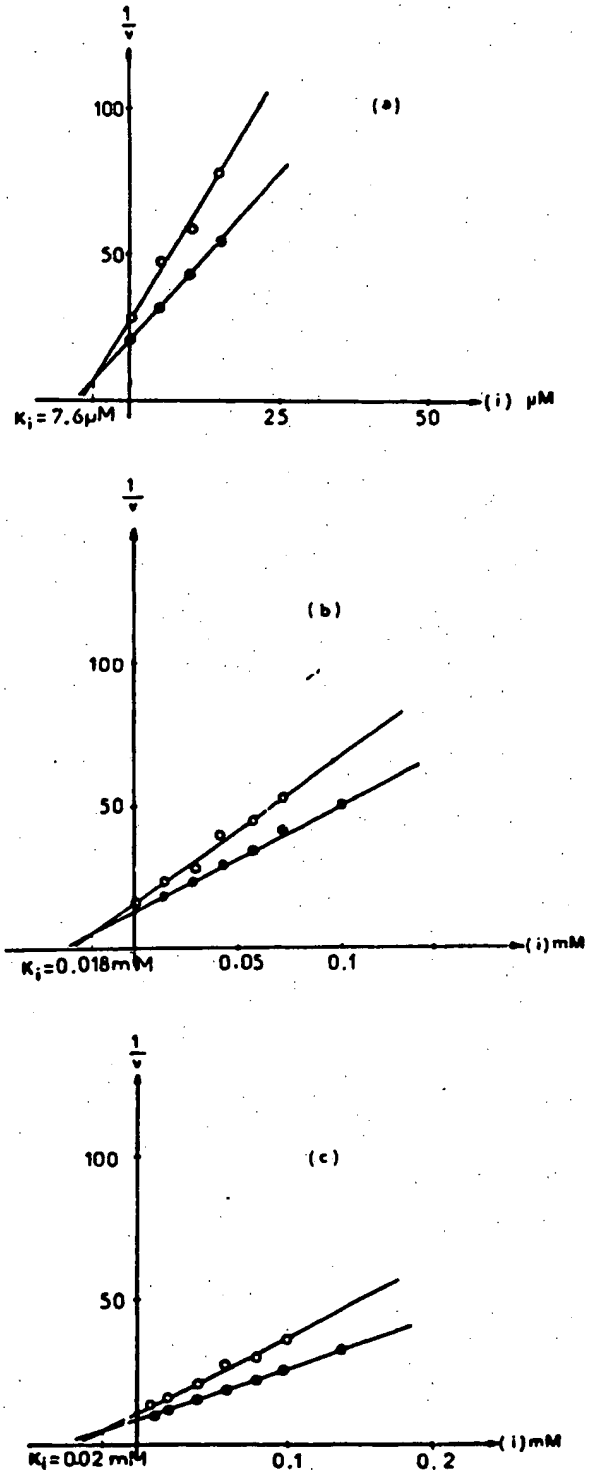

Fig. 3. The competitive inhibition of trypsin by PEGPAB: (a) PEG200PAB, (b) PEG2000PAB, (c) PEG9000PAB. The concentration of DL-benzoylarginine p-nitroanilide was 2.3×10^{-4} M in each of the upper curves and 3.3×10^{-4} M in each of the lower curves. Trypsin concentration, 7×10^{-7} M; 0.05 M Tris buffer; pH 8.15.

that the positive charge of the PAB still exists in the three new inhibitors. It is known that the effectiveness of cationic compounds as trypsin inhibitors is based to a large degree on the attraction of the positive charge to the anionic site of the enzyme (6).

Under our experimental conditions ($K_s = 10^{-3}$ M [5] and $K_i \simeq 10^{-5}$ M) in eq. (5) becomes

$$K_i' \gg 10^{-7}\ \text{M} \qquad (8)$$

As $K_i \simeq 10^{-5}$ M and as the same inhibitor is bound at each end of the PEG chain, it is obvious that eq. (8) is always satisfied.

Thus, a symmetrical arrangement of the inhibitory group at each end of the PEG leads to a simple inhibitor. In other words, nearly no enzyme bound EI to yield E_2I. Geratz and Whitmore [8] reported a similar conclusion with diamidino-α,ω-diphenoxy-alkanes. These compounds function as reversible inhibitors of thrombin, pancreatic kallikrein and trypsin, and each molecule appears to bind only a single enzyme molecule.

PEGPAB have been used to investigate proteins partition in two-phase systems obtained by mixing dextran and PEG solutions. The results will be published in a following paper.

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PARTITION OF TRYPSIN IN TWO-PHASE SYSTEMS CONTAINING A DIAMIDINO-α, ω-DIPHENYLCARBAMYL POLY (ETHYLENE GLYCOL) AS COMPETITIVE INHIBITOR OF TRYPSIN

Georges TAKERKART*, Emile SEGARD** and Michel MONSIGNY*‡

*Centre de Biophysique Moléculaire, CNRS, et Laboratoire de Chimie Biologique, UER Sciences 45045 Orleans-Cedex, France, and
**Centre d'Etudes Cryogéniques de L'Air Liquide 38360 Sassenage, France

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1. Introduction

The purification of biological macromolecules by partition in aqueous two-phase systems is based mainly on physico-chemical properties of systems and of macromolecules. This is well illustrated by the system obtained by mixing aqueous solutions of dextran and poly (ethylene glycol) (PEG). Partition of macromolecules in this system depends namely on polyelectrolytes and salts concentration [1,2]. Furthermore, when charged poly(ethylene glycol) is added, partition depends also on the pH of the system, and proteins can be extracted according to their isoelectric points [3,4]. Interactions involved in the process of partition are not specific and a mixture of proteins is often obtained in each phase of the system. Therefore, it would be of interest to introduce highly specific ligands confined to one of the phases in order to purify macromolecules selectively by affinity.

For enzyme purification, the interacting compound may be a highly specific competitive inhibitor attached to PEG, since it is known that, in dextran/PEG systems, PEG is essentially in the upper phase. As a model, p-aminobenzamidine (PAB), a strong inhibitor of trypsin, was attached to PEG and new macro-inhibitors of trypsin were obtained [5]. We describe here the effect of one of them, PEG 9000 PAB, on the partition of trypsin in dextran/PEG 9000 two-phase system.

‡ To whom inquiries should be addressed.

Abbreviations: ATEE, *N*-acetyl-L-tyrosine ethyl ester.

2. Materials and methods

Dextran T 500 (weight average mol.wt. ($\bar{M}_w$) = 450 000 and number average mol.wt. ($\bar{M}_n$) = 194 000) was supplied by Pharmacia, Uppsala, Sweden. Poly(ethylene glycol) (PEG 9000; $\bar{M}_w$ = 9000) was obtained from Fluka. Diamidino-α,ω-diphenylcarbamyl-poly (ethylene glycol) (PEGPAB): $\bar{M}_w \cong$ 9000) was prepared as described earlier [5] (diamidino-α,ω,-diphenylcarbamyl-poly (ethylene glycol). Trypsin, chymotrypsin, DL-benzoylarginine-*p*-nitroanilide (BAPNA) and *N*-acetyl-L-tyrosine ethyl ester (ATEE) were obtained from Sigma.

2.1. *Phase diagrams*

The binodial for the dextran–PEG–water and for the dextran–PEG/PEGPAB (3:1)–water systems was obtained according to Johansson [3]. Water was added to mixtures of PEG and dextran solutions at room temperature until phase transition occurred.

2.2. *Two-phase systems*

These were prepared at room temperature in 0.05 M Tris–HCl buffer pH 8.0. System (A) was obtained by mixing 20% (w/v) solutions of dextran and PEG

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with buffered enzyme solutions. System (B) was obtained as system (A) but with PEG/PEGPAB (3:1). This ratio was selected to obtain full complexation of trypsin in regard to K_i value of PEG 9000 PAB ($2.0\ 10^{-5}$ M [5]) and concentration of trypsin in the system (10^{-4} M). Increasing concentration of PEGPAB is not useful since solubility of trypsin in the upper phase is limited. The systems were shaken and then centrifuged for 5 min at 1500 *g*. The phase volume ratio is about 2.

2.3. *Determination of partition coefficients*

The partition coefficient of PEG 9000 was determined by evaporating to dryness an aliquot of the upper phase of system (A). The residue was further dried in vacuo over P_2O_5 to constant weight. The partition coefficient of PEG 9000 PAB was determined by measuring the absorbance of each phase at 270 nm with a Zeiss spectrophotometer PMQ II.

2.4. *Determination of trypsin and chymotrypsin activity*

Trypsin assays were carried out with DL-BAPNA as substrate [6,7]. Chymotrypsin assays were carried out with ATEE as substrate [8]. All measurements were made at 25°C using a Beckman Acta III spectrophotometer.

2.5. *Partition of trypsin and chymotrypsin*

Partitions of trypsin and chymotrypsin were studied by measuring enzymic activities in the upper phase and then expressing them in % of total introduced enzyme activities. PEGPAB does not affect the measurement of trypsin activity since its concentration in the sample cuvette (0.01 mM) is very low in regard to that of substrate (1 mM).

3. Results and discussion

3.1. *Phase diagram*

Fig. 1 shows the phase diagrams for system (A) and system (B). The binodial obtained when PEG PAB is added instead of PEG is close to that obtained with unsubstituted PEG. This indicates that the molecular weight of PEG has not changed during substitution [1] in good agreement with our previous results [5].

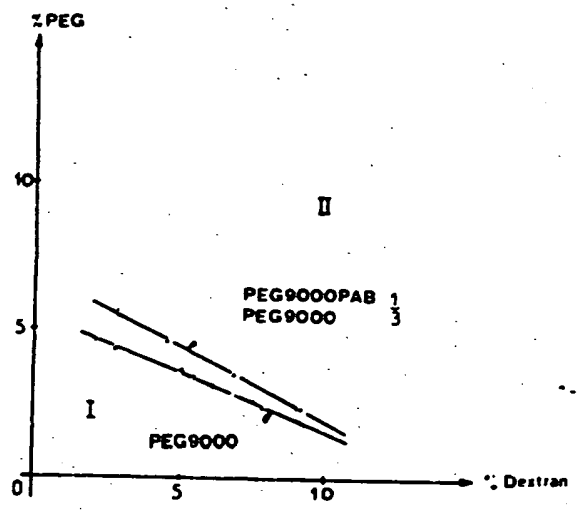

Fig. 1. Phase diagrams for systems PEG9000–dextran–water and PEG9000/PEG9000PAB (3:1)–dextran–water. Part of the binodial separating 1-phase (I) region and the 2-phase region is shown.

3.2. *Partition coefficients of polymers*

The determination of PEG 9000 partition coefficient was conducted as described above because no precipitation was obtained when adding ethanol to an upper phase of system (A). This demonstrates the absence of dextran in the upper phase. The determined value of PEG 9000 partition coefficient is 1.8. In system (B), the ratio of PEGPAB concentrations between the upper and lower phases is 8.6 as determined by spectrophotometric measurements. This value is quite different from that of unsubstituted PEG and indicates an enhancement of the affinity of PEG for the upper phase when *p*-aminobenzamidine is attached to each end of the chain.

3.3. *Trypsin partition*

Concentration of trypsin in systems (A) and (B) was 10^{-4} M. In system (A), recovery of trypsin activity in the upper phase was 40% of the total activity. Partition coefficient of trypsin in that system was calculated to be 0.33. In system (B), recovery of trypsin activity in the upper phase was 92% of the total activity. In that system the trypsin partition coefficient was 5.7. It appears from these values that trypsin concentration in the upper phase is increased as much as 2.3 times when PEGPAB is added to the two-phase system. In other words, there is a 17-fold

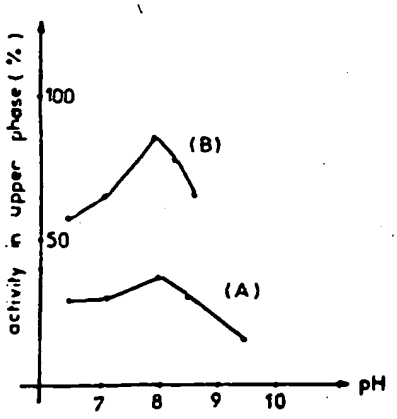

Fig. 2. pH dependance of trypsin partition in dextran–PEG 9000 system (A) and in dextran–PEG9000/PEG9000PAB (3:1) system (B). Trypsin activity is expressed as percentage of total added activity.

increase of the trypsin partition coefficient when a specific trypsin inhibitor is attached to PEG. Thus, the partition coefficient of trypsin is close to that of PEGPAB showing a strong interaction between enzyme and inhibitor.

The pH dependance of trypsin partition in systems (A) and (B) is shown in fig. 2. In both systems, the trypsin partition coefficient exhibits a maximum at pH 8.0. The ionic strength dependence of trypsin partition coefficient is shown in table 1. In system (A), the recovery of trypsin in the upper phase decreased slightly when Tris concentration increased. In system (B), no significant effect could be noted.

Finally, specificity of the extraction of trypsin was demonstrated by investigating partition of chymotrypsin in the same systems. When chymotrypsin was added as a unique protein to systems (A) and (B), recovery of activity in the upper phase was respectively 20% and 28% of total introduced activity. Thus, the chymotrypsin partition coefficients are 0.12 and 0.19 respectively, that is, a 1.5 time increase in system (B). This is to be compared with the 17 times increase obtained with trypsin. Upon addition of a mixture of trypsin and chymotrypsin to systems (A) and (B), the trypsin partition coefficient became 0.26 and 3.35 respectively whereas those of chymotrypsin became 0.11 and 0.15. Though there is a small decrease in trypsin partition coefficients values, PEGPAB still exhibits a highly specific effect.

Table 1
Ionic strength (μ) dependence of trypsin partition in dextran–PEG9000 (system A) and in dextran–PEG9000/PEG9000PAB (3:1) (system B). Tris–HCl buffer pH 8.0

	Trypsin activity in upper phase (in % of total activity)	
μ	System (A)	System (B)
0.05	39	89
0.1	36	77
0.2	35	89

This behaviour is remarkable in consideration of very similar properties of trypsin and chymotrypsin.

This effect seems to be applicable to other interacting systems and its usefulness as a step in macromolecules purification is under investigation.

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